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# Pathobiology of H5N2 Mexican Avian Influenza Virus Infections of Chickens

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**Abstract.** To determine the association between specific structural changes in the hemagglutinin gene and pathogenicity of avian influenza viruses (AIVs), groups of 4-week-old White Plymouth Rock chickens were inoculated intravenously or intranasally with AIVs of varying pathogenicities isolated from chickens in central Mexico during 1994–1995. Mildly pathogenic (MP) viruses had a common hemagglutinin-connecting peptide sequence of Pro-Gln-Arg-Glu-Thr-Arg↓Gly and had restricted capability for replication and production of lesions in tissues. The principle targets for virus replication or lesion production were the lungs, lymphoid organs, and visceral organs containing epithelial cells, such as kidney and pancreas. Death was associated with respiratory and/or renal failure. By contrast, highly pathogenic (HP) AIVs had one substitution and the addition of two basic amino acids in the hemagglutinin connecting peptide, for a sequence of Pro-Gln-Arg-Lys-Arg-Lys-Thr-Arg↓Gly. The HP AIVs were pantropic in virus replication and lesion production ability. However, the most severe histologic lesions were produced in the brain, heart, adrenal glands, and pancreas, and failure of multiple critical organs was responsible for disease pathogenesis and death. No differences in lesion distribution patterns or in sites of AIV replication were evident to explain the variation in mortality rates for different HP AIVs, but HP AIVs that produced the highest mortality rates had more severe necrosis in heart and pancreas. The ability of individual HP AIVs to produce low or high mortality rates could not be explained by changes in sequence of the hemagglutinin-connecting peptide alone, but probably required the addition of other undetermined genomic changes.

*Key words:* Avian species; chicken; encephalitis; immunohistochemistry; influenza virus; myocarditis; pathogenesis; viral diseases.

Avian influenza (AI) is caused by a type A orthomyxovirus. Infections in domestic poultry result in diverse clinical syndromes of varying severity, i.e., differing pathogenicities. The production of a specific disease syndrome or the degree of disease severity depends on multiple factors, including the virulence of the avian influenza virus (AIV) isolate, the presence of suboptimal management conditions, dietary factors, stress, secondary bacterial infections, the host species, the age of the host, and the route of inoculation.[6,28,40] In the field, some H5 and H7 AIVs have been associated with severe fatal systemic disease and have been categorized as highly pathogenic (HP) because they produced high mortality rates in chicken intravenous (IV) pathogenicity tests. By contrast, most H5 and H7 AIVs, and all AIVs of the other 12 hemagglutinin subtypes (H1-4, H6, and H8-14), have been associated with subclinical infections or mildly to moderately severe disease affecting the respiratory, reproductive, and urinary systems.[2,12,17,33] These AIVs have been categorized as nonpathogenic (NP) or mildly pathogenic (MP) because they produced no or few deaths in chicken IV pathogenicity tests, respectively.

Molecular genetic and epidemiologic evidence suggests that most HP AIVs emerge in nature after field passage of NP or MP AIVs in a galliforme host and that they resulted from specific genomic mutations that altered the hemagglutinin surface glycoprotein.[11,19] For example, in 1993, AI appeared in commercial poultry flocks of central Mexico in association with low to moderately high mortality rates, mild respiratory disease, and drops in egg production.[31] These viruses produced no or few deaths in chicken IV pathogenicity tests and were categorized as NP or MP, respectively.[16,39] In the late fall of 1994, in the Mexican state of Puebla, and again in the early winter of 1995 in Queretaro, the AI outbreak changed abruptly, with reports of moderate to high rates of mortality.[31] Chickens that died had gross lesions compatible with previous reports of HPAI[9] and included hemorrhage, edema, and necrosis of combs and wattles; hemorrhages of leg shanks; and serosa of multiple visceral organs.[31] Genetically, these new AIV isolates from Puebla and Queretaro had one substitution and the addition of two basic amino acids at the proteolytic cleavage site of the hemagglutinin, which was consistent with other HP AIVs.[10,11,16] However, the HP Queretaro and Puebla isolates were of different viral lineages (Jalisco and

Puebla, respectively),[11] and the Queretaro isolates were highly lethal, while the Puebla isolates were nonlethal to moderately lethal.[16,37,39] This phenomenon of pathogenicity shifts from low to high has been reproduced in the laboratory with in vivo,[5,8] in vitro,[29] and in ovo[7,15] modeling systems. Such pathogenicity shifts are generally attributed to genomic instability and the lack of polymerase proofreading during virus replication.

The purpose of this study was to identify pathobiologic changes associated with natural and laboratory-induced molecular alterations of the hemagglutinin gene of H5N2 Mexican AIVs. Specifically, the study examined the severity and distribution of gross and microscopic pathologic changes in experimentally infected chickens and identified the organ and cell type of AIV replication. Virologic aspects of the study were reported previously.[37]

## Material and Methods

### Animals and housing

Four-week-old white Plymouth Rock (WPR) chickens were obtained from specific pathogen–free stocks maintained at Southeast Poultry Research Laboratory. All chickens were housed in negative-pressure stainless steel isolation cabinets with continuous light exposure. Water and feed were provided *ad libitum*. All experiments were accomplished in a biosafety level 3 agriculture facility at the Southeast Poultry Research Laboratory and under the guidance of the Institutional Laboratory Animal Care Committee.

### Viruses

Four H5N2 influenza viruses—A/chicken/Hidalgo/26654-1368/94 (H5/94), A/chicken/Jalisco/14589-660/94 (J12/94), A/chicken/Puebla/8623-607/94 (P11/94B), and A/chicken/Queretaro/14588-19/95 (Q1/95)—were isolated from commercial poultry in central Mexico (kindly provided by J. Pearson and D. Senne, Diagnostic Virology, National Veterinary Services Laboratory, US Department of Agriculture). H5/94, J12/94, and Q1/95 were of the Jalisco lineage, and P11/94B was of the Puebla lineage, as determined by nucleotide sequence homology of the HA1 segment of the hemagglutinin gene.[11] H5/94 and J12/94 had a hemagglutinin-connecting peptide sequence of Pro-Gln-Arg-Glu-Thr-Arg↓Gly, while P11/94B and Q1/95 had one substitution and the addition of two basic amino acids in the hemagglutinin-connecting peptide, for a sequence of Pro-Gln-Arg-Lys-Arg-Lys-Thr-Arg↓Gly.[11] Parent stocks, 14-day embryo derivatives (ED), and hen-passed viruses were obtained and used.[37] The second 10-day embryo passage was used as the inoculum for these studies.

### Experimental design

Details of the animal experimentation have been described elsewhere.[37] Briefly, each AIV isolate was inoculated into 4-week-old WPR chickens using a standard pathogenicity test as proposed by the US Animal Health Association.[43] For the four parent stocks of AIV isolates, two replicates of eight chickens each were inoculated intravenously with 0.2 ml of a 1 : 10 dilution of a bacteria-free, infectious allantoic fluid.[43] Hen-passed (P11/94B) and ED (H5/94, J12/94, and P11/94B) AIVs were tested in a standard IV pathogenicity test of eight chickens per isolate. Both H5/94 and J12/94 isolates produced ≤75% mortality and had only two basic amino acids at the proteolytic cleavage site of the HA, supporting categorization as an MP AIV isolate.[37] Q1/95 AIV isolate killed ≥75% of chickens (highly lethal) and was categorized as HP.[37] Parent stock and ED isolates of P11/94B produced ≤75% mortality, (not highly lethal[37]), but the presence of five basic amino acids at the proteolytic cleavage site of the HA suggested the potential for high pathogenicity and warranted categorization as HP.[10,16] Most hen-passed P11/94B AIV isolates were highly lethal and were categorized as HP.[37] In addition to the standard test, the parent stocks were tested in two replicates of eight chickens each in a modified pathogenicity test that substituted intranasal (IN) for IV inoculation.

All chickens that died were necropsied and details of gross lesions were recorded. Tissue samples were taken from one to four chickens per group for histopathology and immunohistochemistry.

### Histopathology and immunohistochemistry

Tissues were saved in 10% neutral-buffered formalin solution. Tissues were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. Unstained paraffin-embedded sections were immunohistochemically stained to demonstrate influenza A nucleoprotein using a described method previously,[34] with some modifications.[36] The primary antibody was a monoclonal antibody against type A influenza virus nucleoprotein (U.S. Hinshaw, College of Veterinary Medicine, University of Wisconsin–Madison).[45] Modifications to the published procedure included adding an antigen retrieval step (Antigen Retrieval Citra Solution, BioGenex, San Ramon, CA), using the primary antibody at 1 : 2000 dilution, using an improved biotin-streptavidin detection system (Super Sensitive Multilink Immunodetection System, BioGenex) and using Fast Red TR system (BioGenex) as the chromogen.

## Results

### Gross pathology

MP H5/94 and J12/94 AIVs produced similar gross lesions in chickens, including mild to severe enlargement of kidneys (86%) with pale pink coloration (64%), urate deposits in ureters and parenchyma (50%), and prominent swelling of renal lobules as seen on the capsular surface (50%); mucus in sinuses and pharynx (50%); mild to moderate enlargement and pale pink coloration of spleens (50%); mild to moderate atrophy of cloacal bursae (36%) and thymuses (21%); fluid or mucus accumulation in the crops (14%); and serosal deposition of visceral urates (7%) on liver, heart, and kidney ("visceral gout"). All HP P11/94B AIV isolates produced lesions similar to those produced by MP AIVs, except atrophy in cloacal

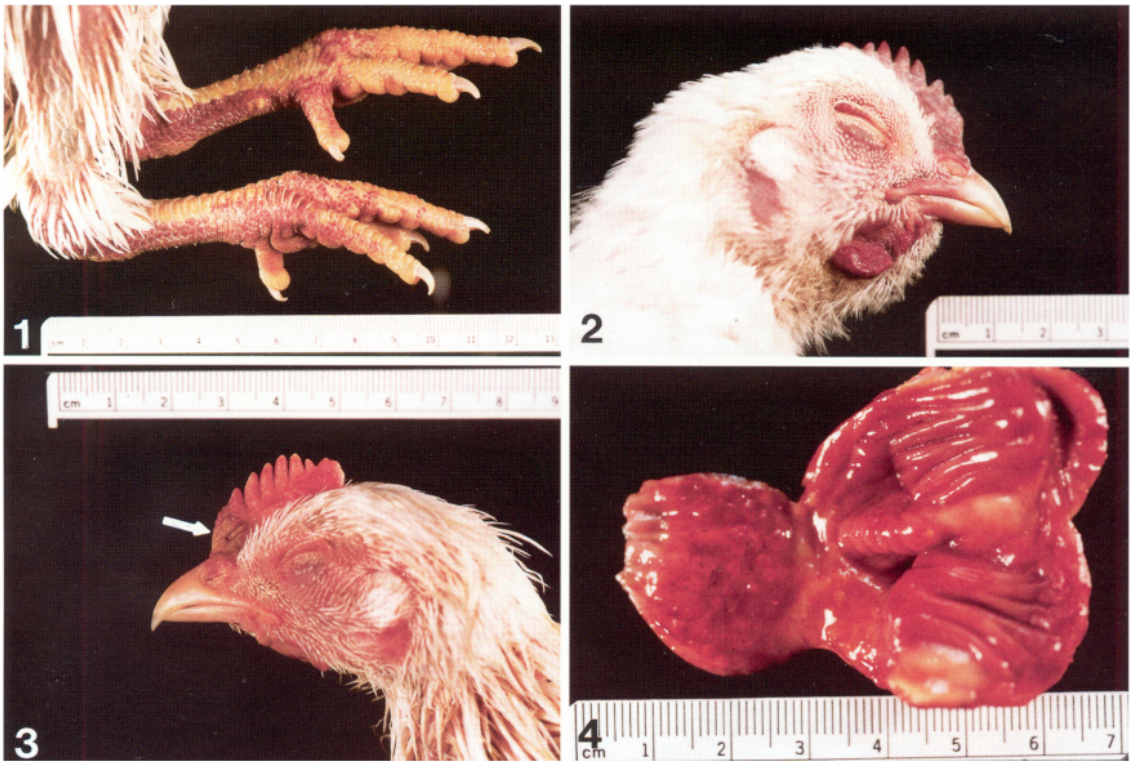


**Fig. 1.** Leg, 4-week-old chicken inoculated intravenously 4 days previously with HP P11/94B AIV derivative. Severe subcutaneous suffusive hemorrhage of leg shanks.

**Fig. 2.** Head, 4-week-old chicken inoculated intranasally 3 days previously with HP Q1/95 parent stock AIV. Severe swelling of the head, comb, and wattles from subcutaneous edema.

**Fig. 3.** Head, 4-week-old chicken inoculated intravenously 4 days previously with HP P11/94B derivative AIV. Large focus of necrosis in the comb (arrow).

**Fig. 4.** Proventriculus and ventriculus, 4-week-old chicken inoculated intranasally 3 days previously with HP Q1/95 parent stock AIV. Severe mucosal hemorrhage in the proventriculus.

bursae and thymuses was more severe with P11/94B AIVs, some P11/94B-infected chickens had subcutaneous congestion and hemorrhage of leg shanks (28%) (Fig. 1) and head (17%), and cloacae were dilated with accumulation of fluid, urates, and bile (17%). Furthermore, hen-passed P11/94B isolates produced petechial hemorrhages in proventriculus (26%), intestines (15%), ventriculus (4%), brain (4%), and other organs (<4%). HP Q1/95 produced lesions similar to those produced by hen-passed P11/94B AIVs, but in addition, mild to severe subcutaneous edema was common in the head (56%) including the comb and wattles (Fig. 2), over the keel (31%), and in the distal legs, proximal to tibiotarsal joint (72%); and necrosis was present in the combs and wattles. A few chickens infected with hen-passed P11/94B AIV had necrosis of the comb (4%) (Fig. 3) and wattles (1%). In Q1/95-infected chickens, atrophy in cloacal bursae and thymuses was more frequent (88% and 84%, respectively) and more severe; and hemorrhage of proventriculus (Fig. 4) and ventriculus was more severe than in P11/94B-infected chickens. Hydropericardium was identified infrequently in a few IV-inoculated chickens receiving HP hen-passed P11/94B (1%) and HP parent stock Q1/95 (3%) AIV. IN versus IV routes of inoculation had little impact on frequency and distribution of lesions with any of the four parent stock AIVs.

### Histopathology and immunohistochemistry

HP P11/94B (parent stock, ED and hen-passed) and HP Q1/95 AIVs produced mild to severe multifocal lymphohistiocytic myocarditis (75% and 50% of chickens, respectively) with associated multifocal myocyte degeneration and necrosis (Fig. 5) (Table 1). Some intranuclear inclusion bodies were present in necrotic myocytes. The inflammation was more severe and necrosis less severe with P11/94B than with Q1/95 AIVs (Table 1). Intranuclear and intracytoplasmic staining for AIV nucleoprotein was seen in degenerating and necrotic myocytes (Fig. 6) and in neurons of autonomic ganglia (Table 2). P11/94B produced moderate multifocal lymphocytic meningoencephalitis with

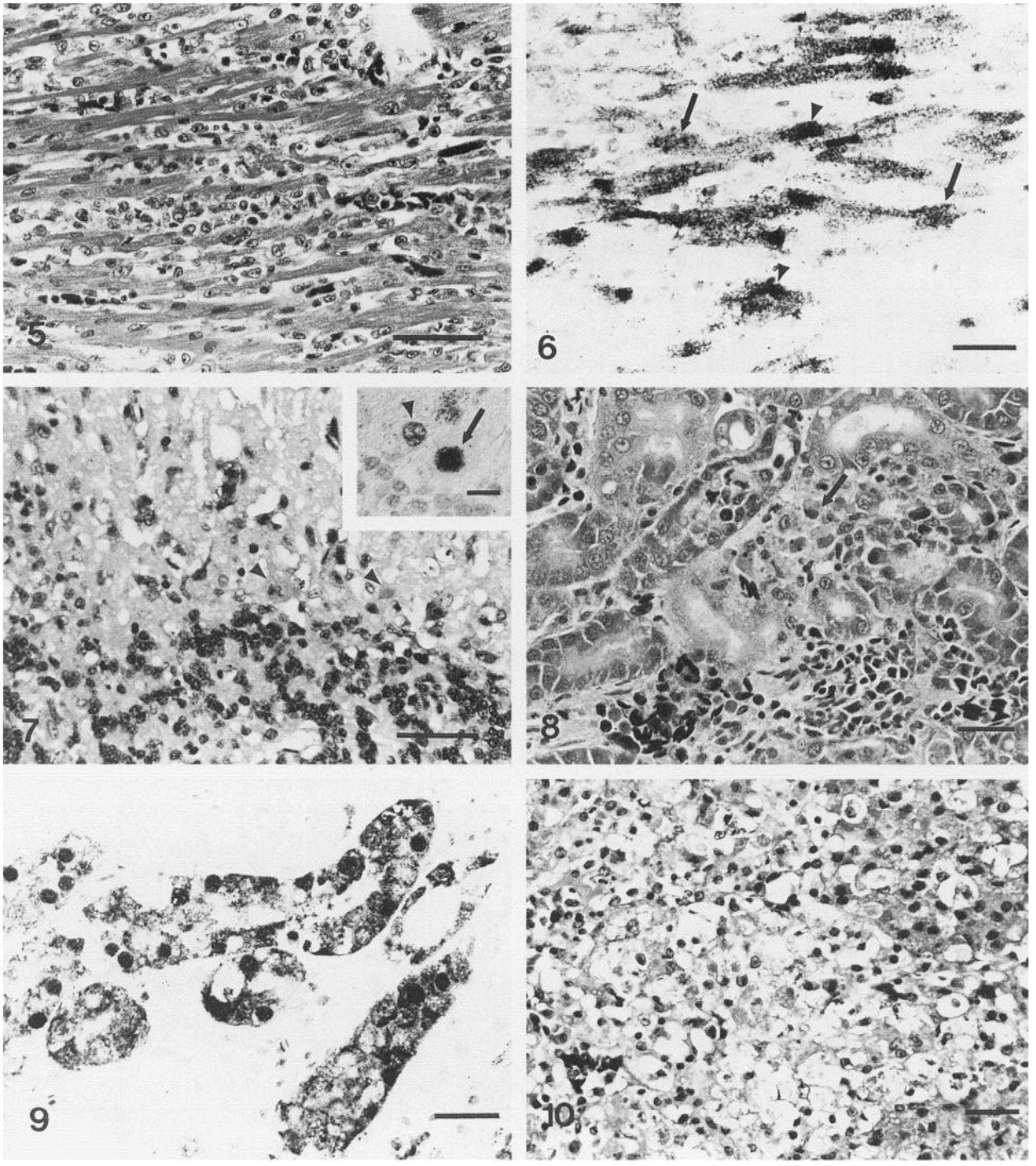


**Fig. 5.** Heart, chicken inoculated intranasally 5 days previously with HP Q1/95 parent stock AIV. Diffuse lymphohistiocytic myocarditis with multifocal degeneration and necrosis of myocytes. HE. Bar = 50 μm.

**Fig. 6.** Heart, chicken inoculated intranasally 5 days previously with HP Q1/95 parent stock AIV. Intranuclear (arrowheads) and intracytoplasmic (arrows) AIV antigen detected in degenerating and necrotic myocytes in areas of myocarditis. Avidin–biotin complex immunoperoxidase, hematoxylin counterstain, differential interference contrast microscopy. Bar = 20 μm.

**Fig. 7.** Cerebellum, chicken inoculated intravenously 5 days previously with HP P11/94B derivative AIV. Vacuolation of the molecular layer with associated mild diffuse gliosis and necrosis of Purkinje neurons (arrowheads). HE. Bar = 50 μm. *Inset:* Intranuclear AIV antigen in a Purkinje neuron (arrow) and an associated Bergmann's glial cell (arrowhead). Avidin–biotin complex immunoperoxidase, hematoxylin counterstain, differential interference contrast microscopy. Bar = 10 μm.

**Table 1.** Most common histopathologic changes* in 4-week-old White Plymouth Rock chickens that died after intravenous or intranasal inoculation with Mexican avian influenza viruses ($n$ = 4–9).

| Tissue/Lesion | Jalisco Lineage | | | Puebla Lineage |
|---|---|---|---|---|
| | MP H5/94 | MP J12/94 | HP Q1/95 | HP P11/94B |
| Heart | | | | |
| Myocyte necrosis | −† | − | +++ | ++ |
| Myocarditis | − | − | ++ | +++ |
| Brain | | | | |
| Neuron necrosis | − | − | +++ | +++ |
| Meningoencephalitis | − | − | + | +++ |
| Kidney | | | | |
| Tubule necrosis | +++ | ++ | + | + |
| Interstitial nephritis | +++ | ++++ | − | +++ |
| Cloacal Bursa | | | | |
| Lymphocyte necrosis | ++ | + | + | + |
| Lymphocyte depletion | +++ | +++ | ++++ | +++ |
| Spleen | | | | |
| Lymphocyte necrosis | + | − | ++ | + |
| Lymphocyte depletion | +++ | +++ | +++ | + |
| Macrophage-phagocyte system hyperplasia | + | − | + | ++ |
| Thymus | | | | |
| Lymphocyte necrosis | + | − | ++ | + |
| Lymphocyte depletion | + | + | ++++ | ++++ |
| Trachea | | | | |
| Deciliation | − | − | + | ++ |
| Inflammation | − | − | − | + |
| Lung | | | | |
| Interstitial pneumonia | +++ | ++ | +++ | + |
| Adrenal | NE‡ | | | NE |
| Degeneration/necrosis of corticotrophic cells | | − | ++ | |
| Degeneration/necrosis of chromaffin cells | | − | + | |
| Pancreas | | | | |
| Vacuolation | + | ++ | +++ | +++ |
| Necrotic acinar epithelium | − | − | +++ | ++ |
| Cecal tonsil | | | | |
| Hemorrhage | − | − | − | ++ |
| Lymphocyte depletion and necrosis | − | − | +++ | +++ |

* No lesions in duodenum, jejunum, liver, or thyroid gland.
† − = No or sporadic lesions; + = minimal; ++ = mild; +++ = moderate; ++++ = severe lesion.
‡ NE = not examined.

prominent gliosis (Fig. 7). Q1/95 AIVs produced little or no inflammatory lesions in the brain. With both HP viruses, neuronal necrosis was widespread, especially in Purkinje neurons, but was also present in neurons of the granular cell layer, cerebral hemispheres, and medulla oblongata. Intranuclear eosinophilic inclusion bodies were identified occasionally in Purkinje neurons, and the inclusions stained positive for influenza

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**Fig. 8.** Kidney, chicken inoculated intravenously 3 days previously with MP H5/94 parent stock AIV. Necrotic tubule (arrow) with heterophilic-to-lymphocytic interstitial nephritis. HE. Bar = 25 μm.

**Fig. 9.** Kidney, chicken inoculated intravenously 3 days previously with MP H5/94 parent stock AIV. Intranuclear and intracytoplasmic AIV antigen in degenerate and necrotic proximal tubule epithelium. Avidin–biotin complex immunoperoxidase, hematoxylin counterstain, differential interference contrast microscopy. Bar = 20 μm.

**Fig. 10.** Adrenal, chicken inoculated intravenously 4 days previously with HP Q1/95 parent stock AIV. Severe multifocal necrosis of corticotrophic cells. HE. Bar = 25 μm.

**Table 2.** Distribution of influenza A nucleoprotein* as demonstrated by immunohistochemistry in tissues from 4-week-old White Plymouth Rock chickens that died after inoculation with Mexican avian influenza viruses ($n$ = 4–9).

| Tissue | Jalisco Lineage | | | Puebla Lineage |
|---|---|---|---|---|
| | MP H5/94 | MP J12/94 | HP P11/94B | HP Q1/95 |
| Heart | | | | |
| Myocytes | −† | − | +++ | ++ |
| Neurons of autonomic ganglia | − | − | + | − |
| Brain | | | | |
| Neurons | − | − | +++ | +++ |
| Ependymal cells/choroid plexus | − | − | + | + |
| Glial cells | − | − | + | + |
| Kidney | | | | |
| Tubule epithelium | +++ | ++ | + | + |
| Cloacal bursa | | | | |
| Lumenal epithelium | + | − | − | − |
| Macrophages in medulla | + | − | + | + |
| Spleen | | | | |
| Ellipsoid capillary endothelium | − | − | + | + |
| Macrophages and necrotic debris | − | − | + | − |
| Thymus | | | | |
| Thymic epithelium | − | − | + | + |
| Trachea | | | | |
| Respiratory epithelium | − | − | + | + |
| Lung | | | | |
| Air/blood capillary endothelium | − | − | + | + |
| Macrophages in air capillary walls | − | − | − | + |
| Adrenal | NE‡ | | | NE |
| Corticotrophic cells | | − | +++ | |
| Chromaffin cells | | − | + | |
| Duodenum | | | | |
| Crypt epithelium | − | − | + | − |
| Myenteric ganglial neurons | − | − | + | − |
| Pancreas | | | | |
| Acinar epithelium | − | − | +++ | + |
| Jejunum | | | | |
| Crypt epithelium | − | − | + | − |
| Myenteric ganglial neurons | − | − | + | − |
| Cecal tonsil | | | | |
| Crypt epithelium | − | − | − | + |
| Myenteric ganglial neurons | − | − | + | − |
| Macrophages in lamina propria | − | − | + | − |
| Liver | | | | |
| Sinusoidal epithelium, Kupffer cells | − | − | + | − |

* No antigen was detected in the thyroid gland.
† − = none; + = infrequent or rare; ++ = frequent; +++ = common; ++++ = widespread.
‡ NE = not examined.

nucleoprotein (Fig. 7, inset). Influenza nucleoprotein was common in nuclei and cytoplasm of Purkinje neurons and less common in neurons of medulla oblongata and cerebral hemispheres (Table 2). Influenza nucleoprotein was infrequent in ependymal cells, especially of lateral ventricles, Bergmann's glial cells, astrocytes, and choroid plexus epithelium (Table 2). Nucleoprotein demonstration in the brain was more frequent in chickens inoculated by the IV than by the IN route with Q1/95 AIV. Chickens infected with MP H5/94 and J12/94 AIVs lacked lesions and nucleoprotein staining in heart and brain samples (Tables 1 and 2).

Tubule necrosis and associated heterophilic-to-lymphocytic interstitial nephritis was present in all chickens infected with MP H5/94 and J12/94 AIVs. The kidney lesions were moderate to severe (Table 1) (Fig.

8). In addition, an MP H5/94 IN-inoculated chicken that died had widespread deposition of needle-shaped urate crystals in or on most visceral organs, including the kidneys. For HP P11/94B and Q1/95 viruses, necrosis of tubules was minimal (Table 1). There was no inflammation in kidneys from chickens infected with Q1/95 AIV, but inflammation was moderately severe in kidneys infected with P11/94B. Intranuclear and intracytoplasmic staining of kidney tubules for nucleoprotein (Fig. 9) was frequent to common with H5/94 and J12/94 AIVs but was infrequent or rare with P11/94B and Q1/95 AIVs (Table 2).

In primary (thymus and cloacal bursa) and secondary lymphoid organs (spleen, gut-associated lymphoid tissue, and cecal tonsil), MP H5/94 and J12/94 AIVs produced minimal to moderate lymphocyte depletion and some lymphocyte necrosis in 93% of the chickens, but AIV nucleoprotein was rarely demonstrated in lymphoid organs and was not seen in lymphocytes (Tables 1 and 2). Occasionally, AIV antigen was demonstrated in lumenal epithelial cells and medullary macrophages of the cloacal bursa. Lymphocyte depletion and necrosis were more severe for HP P11/94B and Q1/95 AIVs, compared to MP AIVs, especially in the thymus and cecal tonsil samples. With both P11/94B and Q1/95 AIVs, nucleoprotein was usually localized to capillary endothelium, macrophages, and thymic epithelium of lymphoid organs.

Mild to moderate interstitial pneumonia was a common lesion in chickens infected with MP H5/94 (78%), MP J12/94 (100%), HP P11/94B (48%), and HP Q1/95 (88%) AIVs (Table 1). The predominant inflammatory cell type was the macrophage, but heterophils were common. Necrosis of air and blood capillary endothelium was present in lungs of some chickens inoculated with HP AIVs. With all four AIVs, the interstitial pneumonia was diffuse and more common in IV-inoculated chickens, and it was focal and less common in IN-inoculated chickens. Tracheas from 17% of chickens inoculated with HP AIVs had mild lesions, including deciliation, lymphocytic tracheitis, and accumulation of fibrinoheterophilic exudate within the lumen. Nineteen percent had infrequent or rare demonstration of influenza nucleoprotein in tracheal epithelial cells. Tracheas from chickens inoculated with MP AIVs lacked lesions and influenza viral antigen.

Endocrine glands varied in the presence or absence of lesions (Table 1). Thyroid glands from all virus groups lacked lesions, and AIV nucleoprotein was not demonstrated. Adrenal glands from chickens infected with HP Q1/95 had degeneration and necrosis of corticotrophic and, less frequently, chromaffin cells (Fig. 10). AIV nucleoprotein was consistently localized to necrotic corticotrophic cells, but only occasionally to chromaffin cells (Table 2). MP J12/94 AIV did not produce lesions in adrenal glands, and nucleoprotein was not demonstrated.

Moderate to severe pancreatic lesions, especially necrosis, were identified in chickens infected with P11/94B (85%) and Q1/95 AIVs. Nucleoprotein was commonly identified in necrotic acinar epithelium. MP AIVs produced minimal to mild vacuolar degeneration in pancreatic acinar epithelium in a few chickens (18%), but without necrosis and inflammation. For all virus groups, inflammatory or necrotic lesions were rare or nonexistent in duodenum, jejunum, and liver.

When results were compared for routes of inoculation and virus source for HP P11/94B, histopathologic changes varied in severity. The presence of necrosis and detection of nucleoprotein was greater in both kidney tubule and pancreatic acinar epithelium of chickens inoculated intranasally with P11/94B AIV than in chickens inoculated intravenously with P11/94B AIV. IV inoculation with ED-7, 9 and 23 P11/94B AIVs produced lesion severity and antigen distribution patterns similar to those produced by the parent stocks and had moderately high mortality rates. However, the hen-passed P11/94B isolates produced high lethality rates, more severe myocyte degeneration and necrosis in heart, and more severe necrosis of pancreatic acini.

## Discussion

Distribution and severity of lesions in chickens that died after inoculation with Mexican H5N2 AIV isolates varied greatly between the MP and HP categories of viruses. The MP AIVs had restricted capability for replication and production of lesions in individual cell types, tissues, and organs. The principle targets for virus replication, as supported by immunohistochemical demonstration of AIV antigen or histologic lesion production, were the lungs, lymphoid tissues, and visceral organs containing epithelial cells, such as kidney and pancreas. By comparison, HP AIVs were pantropic in virus replication and ability to produce gross and histologic lesions. However, the most severe lesions of necrosis, hemorrhage, and inflammation, as evident on gross and microscopic examination, were produced in the skin, brain, heart, adrenal glands, and pancreas. Such differences in tissue tropism for MP and HP AIVs reflect varying pathogenic mechanisms involved in the production of clinical illness and death of infected chickens and were associated with differences in the proteolytic cleavage site of hemagglutinin surface glycoprotein.[37]

Most NP/MP AIVs, including H5/94 and J12/94 isolates of the current study, have two basic amino acids at the proteolytic cleavage site of the hemagglutinin protein[48] and require cleavage by a trypsin-like enzyme to be infectious and accomplish multiple virus

replication cycles.[22] Typically, NP/MP AIVs have been isolated from respiratory secretions and feces of naturally or experimentally infected poultry, and AIV nucleoprotein has been identified in epithelial cells of the intestine, trachea, lungs, and airsacs.[32,34,35,38,41] These organs have trypsin like enzyme activity in epithelial cells[21] or within lumenal contents, and such a physiological feature is responsible for the respiratory and gastrointestinal tracts being primary sites for NP/MP AIV replication and lesion production. Occasionally, NP/MP AIVs can spread beyond the respiratory and gastrointestinal tracts, replicate, and produce lesions in specific visceral organs, primarily organs containing epithelial cells, such as kidney and pancreas.[32,37,42] In addition, NP/MP AIVs have produced lymphocyte depletion and necrosis in primary and secondary lymphoid tissues of chickens. However, AIV antigen was not identified in lymphocytes but was found occasionally in cells of the macrophage-phagocytic cell system and surface epithelium of the cloacal bursa. This suggests that depletion and necrosis of lymphocytes was secondary to stress, possibly from increased endogenous glucocorticoids or from secretion of specific cytokines, and not from virus replication in lymphocytes.

Sporadic deaths have resulted from infections with NP/MP AIV in chickens and turkeys, usually between 2 and 7 days after inoculation. In individual birds, AIV replication and accompanying necrosis and inflammation were present in the lungs and kidneys, but virus replication and accompanying less severe lesions were also present in other organs containing epithelial cells, such as pancreas, trachea, and oviduct (current study).[9,32,38,41,42] However, lesions were specifically lacking in brain, heart, vascular tissues, and endocrine organs. Organ involvement in multiple viral replication cycles and lesion production was similar in both IN- and IV-challenge models. Although lung and kidney lesions were more frequent and more severe following IV challenge, the distribution pattern for interstitial pneumonia varied from diffuse in IV-challenge to focal or multifocal around secondary bronchi in IN-challenge models (current study).[32,41] These differences reflect a more severe challenge in the simulated viremia model (IV route of inoculation) versus a less severe challenge in the natural exposure model (IN route of inoculation).

In contrast to NP/MP AIVs, HP AIVs had either additions or substitutions of multiple basic amino acids at the proteolytic cleavage site of the hemagglutinin and/or the loss of a shielding glycosylation site adjacent to the cleavage region.[20,46] For example, HP Q1/95 and P11/94B AIV isolates and derivatives had an insertion of two basic amino acids at the cleavage site and substitution of a basic amino acid at −3 position[11,16,39] and segment, and HP SA/61 had an insert of three additional basic amino acids at the cleavage site, while HP A/chicken/Pennsylvania/1370/83 (H5N2) [HP Pa/83] lacked a glycosylation site in association with a change at residue 13 of the HA1.[19,48] The presence of such molecular features at the proteolytic cleavage site resulted in the activation of the hemagglutinin, by ubiquitous intracellular proteases, and production of infectious virus.[30] These proteases are present in a wide variety of cell types, which resulted in replication of AI virus to high titers, multiple viral replication cycles, production of viremia, and death of a variety of cell types in many different organs. This scenario is common in the pathogenesis of HP AIV infections of poultry, but variation exists in the specific organ, tissue, and cell replicative trophism and pathogenicity of individual AIVs. For example, in the current study, the lesion distribution pattern was pantropic and similar for HP P11/94B parent stock, ED and hen-passed derivatives, and Q1/95 parent stock. However, the mortality rates were highest, gross lesions of subcutaneous and subserosal edema and hemorrhage were more severe, and histologic lesions in the heart and pancreas were more severe in chickens infected with HP hen-passed P11/94B and parent stock Q1/95 AIVs. This suggests that differences in other parts of hemagglutinin or in the other seven genes of AIV impacted disease pathogenesis, especially the production of lesion severity and high mortality rates.

By contrast to NP/MP AIVs, HP AIVs Q1/95 and P11/94B produced severe necrosis and inflammation within the heart, brain, adrenal glands, and pancreas, and less severe necrosis in kidneys. This lesion pattern, excluding the lack of sampling for adrenal, is most similar to patterns observed in the field outbreak in Pennysylvania during 1983 and in chickens inoculated experimentally with A/tern/South Africa/61 (H5N3) [HP SA/61] and HP Pa/83.[1,25,44] Other HP AIV produced some of these systemic lesions, especially involvement of the brain, but specific organ involvement and severity of lesions varied with individual viruses and with different host species. For example, HP A/chicken/Scotland/59 (H5N1) [Sc/59] produced severe brain lesions in IN-inoculated chickens, but infected chickens lacked heart lesions.[44] In naturally infected turkeys, HP A/turkey/England/91 (H5N1) [En/91] produced severe lesions in brain, mild to moderate lesions in pancreas, but infrequent lesions in the heart.[3] An experimental study in chickens with En/91 has confirmed the consistency and severity of pathogenicity for brain and virus replication within neurons.[23] HP A/turkey/Ontario/66 (H5N9) [On/66] produced severe brain lesions and moderate heart and kidney lesions in intratracheal (IT)-inoculated turkeys but either did not produce or inconsistently produced mild lesions in brain, heart, or kidney of IT-inoculated chickens.[25–27]

For HP H7 AIVs, the most consistent lesions were in brain and pancreas of experimentally inoculated chickens, but the lesions varied from minimal to severe in heart, skeletal muscle, and kidney.[14,25,47]

Clinical illness and death in chickens infected with HP AIVs may be the result of multiorgan failure or involvement of a few critical organs in the nervous, cardiovascular, or endocrine systems. Virus replication and lesion development could lead to peracute to acute death with minimal or focal lesions, especially when affecting cardiac muscle or conduction fibers, arterioles or capillary vascular beds, brain (especially respiratory centers), adrenal glands, and pituitary. This could explain deaths within 24–48 hours after inoculation[23] and the presence of meager gross and/or histologic lesions until after 72 hours.[23] However, poultry that survive 2–5 days have had well-developed lesions in the brain, heart, and/or cardiovascular system, and these lesions could be responsible singularly or collectively for illness and death of the birds.

First, the most frequent and severe lesions have been in the brain of chickens infected with HP Q1/95, P11/94B, Pa/83 (H5N2), A/chicken/Victoria/1/85 (H7N7), A/chicken/Victoria/92 (H7N3), En/91 (H5N1), and SA/61 (H5N3) (current study).[1,13,14,23,25,26,44] Such brain lesions were associated with isolation of AIV from the brain[49] and/or detection of AIV proteins in neurons, especially in Purkinje neurons, ependyma, glial cells, and vascular endothelium.[14,23,25] Focal distribution of AIV replication and death of select neurons or the rapid widespread replication and death of brain vascular endothelium with thrombosis could be a major pathogenic mechanism responsible for illness and death.

Second, frequent and severe lesions have been reported in the hearts of chickens infected with several HP H5 and H7 AIVs, and this raises some doubt that neuronal pathogenicity is the only mechanism responsible for illness and death. In the current study, chickens infected with P11/94B parent stock, ED, and hen-passed derivatives had similar distribution pattern for lesions and viral antigen localization in most organs, including the brain, and these viruses had identical cleavage site sequences for the hemagglutinin surface glycoprotein. However, the highest mortality rates occurred in chickens infected with hen-passed P11/94B, and this coincided with demonstration of abundant AIV antigen in cardiac myocytes and necrosis and inflammation in the heart. Similar demonstration of AIV antigen in cardiac myocytes and associated necrosis and inflammation in the heart has been reported for chickens and turkeys infected with HP Pa/83 and On/66, respectively.[4,25] Furthermore, the turkeys infected with HP On/66 had electrocardiographic changes indicating severe cardiac conduction defects as early as 72 hours after inoculation,[24] and these were either the result of direct virus damage to cardiac muscle and conduction fibers or secondary to hyperkalemia from widespread tissue necrosis.[24] These findings suggest that cardiopathogenicity contributes to disease pathogenesis in domestic poultry infected by some HP H5 AIVs.

Third, collapse of the cardiovascular system as the result of widespread vascular endothelial cell necrosis and vasculitis, thrombosis, disseminated intravascular coagulation, and ultimately ischemia could be involved in disease pathogenesis and could be responsible for death in HP AIV-infected chickens.[4,14] In the current study with Q1/95, necrosis of pulmonary capillary endothelium resulted in widespread pulmonary edema and respiratory collapse. Finally, the necrotic lesions in corticotrophic and chromaffin cells of adrenal glands of the current study may indicate a pathophysiologic alteration of endocrine glands in disease pathogenesis of HP AIV. By contrast to HP AIVs, pathologic studies suggest that NP/MP AIVs in poultry caused illness and death by interfering with normal respiratory function or by causing acute renal failure, as evident by hyperuricemia, hypercalcemia, and hyperphosphatemia.[32,38,40,41]

With HP AIVs, kidney lesions were a minor or absent feature,[1,4,18,44] but in other studies, the kidney was a significant site of lesion production and virus replication.[14,25,49] A portion of this variation may be attributed to differences in host age, route of inoculation, and virus inoculum dose. However, in some studies, the lack of kidney lesions in dead birds may be the result of early and rapid virus replication, injury, and death to significant cell populations in critical organs, such as heart, brain, or capillary beds. This would result in peracute death before pathobiological and clinical manifestations of kidney trophism and pathogenicity could occur. Extensive damage to kidneys could result in clinical illness and death if the majority of kidney is acutely involved, as with MP AIVs inoculated by IV route,[40] but the pathogenesis requires a sufficiently long survival period to produce acute to subacute renal failure and death.

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